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In vitro and in vivo chemotherapy screening of the divalent cation chelator 1,10-orthophenanthroline*

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Summary. 1,10-Orthophenanthroline (OP) is a divalent cation chelating agent with known cytotoxicity to human normal and malignant T-lymphocytes. To determine whether OP might be a useful anticancer agent with specific T cell toxicity, OP's effect on cell growth was determined on colony-forming cells. The assay used supported growth of both malignant lymphoid and normal myeloid colony-forming cells (CFU-C) and thus a direct comparison of OP's antilymphoid and antimyeloid toxicity was obtained. The malignant lymphoid cells tested were established from patients at relapse and were resistant to conventional chemotherapeutic agents in vitro.

While OP was found to be toxic to all cells tested, some selective kill of malignant cells over CFU-C occurred. OP's cytotoxicity was time-dependent and a three-log enhanced kill occurred when the drug exposure time was increased from 1 to 24 h. When test cells were continously exposed to OP, the ID_{50} was less than 1 μg/ml for malignant lymphoid cells and the sensitivity index (SI = x ID_{50} CFU-C ÷ x ID_{50} cell line) ranged from 1.5 to 3.0. The National Cancer Institute currently screens new compounds for antitumor activity by determining whether the test drug is toxic to a mouse lymphocytic leukemia cell line (P388). While the mouse P388 cells were sensitive to OP in vitro, no effect was seen when OP was administered in vivo, even when schedules designed to take advantage of OP's time-dependent toxicity were used. Since malignant cells were sensitive to OP (ID_{50} < 1 μg/ml), and some selectivity over CFU-C occurred (SI > 1), OP may be a useful agent for control of leukemic cell growth in vitro. However, since OP did not control the growth of P388 cells in vivo, additional studies designed to enhance the therapeutic index of OP in vivo are needed.

Introduction

1,10-Orthophenanthroline (OP) is a divalent cation chelating agent which causes inhibition of DNA synthesis in human T-lymphocytes [21], the mouse lymphocyte cell line L1210 [2], and the human lymphoblastic T cell line CCRF-CEM [3]. In these cells, OP causes inhibition of zinc-dependent enzymes (i.e., DNA polymerase, thymidine kinase), and the toxic effect of OP can be blocked when supplemental zinc is added to the culture medium [2, 3, 21].

Impaired T-lymphocyte function is a well-recognized sequela of clinical zinc (Zn) deficiency, as seen in patients with acrodermatitis enteropathica [8] or prolonged hyperalimentation without Zn supplementation [11]. In human and animal models, manifestations of T-lymphocyte dysfunction include anergy, thymic atrophy, defective cell-mediated immunity, and decreased T cell response to PHA stimulation [1, 5]. However, granulocytes and B-lymphocytes appear to be spared from the toxic effects of low zinc, because both cell number and cell function are normal in patients and animals with zinc deficiencies [7, 8, 11, 22]. To evaluate whether OP is specifically toxic to T cells, we determined the effect of OP on B and T cell lines and on normal bone marrow colony-forming cells (CFU-C).

Materials and methods

Experimental cells and culture conditions. The human cell lines used in these experiments were established in our laboratory, and the basic cell culture procedures have been previously published [16–18]. All cell lines were grown in suspension culture in modified McCoy 5A medium supplemented with 15% newborn calf serum, penicillin (5 units/cm^3), streptomycin (0.5 μg/cm^3), and *L*-glutamine (40 μ*M*/cm^3) (complete medium, CM). Each cell line was split two or three times per week and was evaluated for drug sensitivity while in log phase growth.

Human myeloid/monocytic colonies (CFU-C) were cultured from surveillance bone marrow aspirates from children with acute lymphoblastic leukemia in remission. Marrow aspirates were taken at a time when the myelosuppressive effects of maintenance chemotherapy had resolved. The protocol procedures were approved by the Medical Committee for the Use of Human Subjects in Research, and informed consent was obtained.

Culture conditions. Bone marrow aspirates were processed for CFU-C growth as previously described [12, 17]. Briefly, after Ficoll–Hypaque separation, mononuclear cells (10^5) were mixed with agar (final concentration 0.3%) and plated onto petri dishes containing a feeder layer. The feeder layer for CFU-C growth contained a mixture of agar (0.5%), CM, plasma (10%), and 10^6 peripheral blood WBCs

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[12, 17]. The cell lines ($1-10\times10^4$ cells/cm^3) were cultured in the same manner except that the feeder layer contained a mixture of agar (0.5%), CM, and human serum (10%). All cultures were incubated in a humidified incubator gassed with 5% CO_2 in air. After 14–18 days of culture, tight cell groups of greater than 50 cells were scored as colonies.

Experimental drugs. 1,10-Orthophenanthroline (Sigma Chemical, St. Louis, Mo) was suspended in CM, warmed to 100 °C for 5 min, filtered, and serially diluted with CM. CaEDTA (Sigma Chemical) was similarly prepared except that heating was unnecessary. For the blockage and rescue experiments, stock solutions of $ZnSO_4$ and $MgSO_4$ (Sigma Chemical) were suspended in distilled water, filtered, and diluted to the test concentration with CM.

Drug exposure. Cells were incubated with test drug (or CM for control) for 1, 4, or 24 h at 37 °C in 5% CO_2 in air. Drug removal was accomplished by dilution with CM (and centrifugation, x3) to dilute the test drug to a concentration below the threshold of toxicity (1:10,000). Cells were then resuspended in an agar–CM mixture and plated. In separate experiments, cells were also exposed to OP continuously throughout colony growth (2 weeks). In these experiments, OP (or EDTA) was added directly to the petri dishes and no drug removal was performed.

Cation rescue and blockage experiments. Media supplemented with varying concentrations of Zn and Mg were tested for their ability to support cell proliferation without toxicity. The maximum nontoxic concentations of supplemented media were found to contain Zn (700 μg/dl) or Mg (30 mg/dl). The OP effect on cell growth was evaluated in: (1) standard complete medium (CM) with baseline concentrations of Zn (35 μg/dl) and Mg (3 mg/dl); (2) CM containing supplemental Zn (700 μg/dl) with Mg (3 mg/dl); and (3) CM containing supplemental Mg (30 mg/dl) with Zn (35 μg/dl). In the cation *blockage* experiments, the test cells were suspended in one test medium, mixed with agar, and plated onto petri dishes. Then OP (or diluent for control) was added to each plate and the cells were exposed to OP continuously for 2 weeks. In the cation *rescue* experiments, the cells were suspended in CM and incubated with OP for 24 h. After washing to remove OP, the cells were suspended in one of the three test media, mixed with agar, and plated. OP was tested at concentrations of 0.6, 1.5, and 2.6 μg/cm^3, corresponding to the ID_{50}, ID_{70} and ID_{90} concentrations, respectively.

In vivo drug screening. The mice (BDF_1) and 5-fluorouracil (5-FU) used in these experiments were obtained from the Developmental Therapeutics Program, National Cancer Institute. NCI protocol 1.200 for the in vivo evaluation of test drugs on P388 lymphocytic leukemia was utilized [4]. Basically, 10^6 P388 cells were inoculated IP on day O and specific therapy was started 24 h later. The drugs were given via the routes according to the treatment schedules itemized in Table 2. Each treated group consisted of 4–9 animals and the untreated control groups consisted of 4–18 animals as per the protocol [4]. Antitumor activity was determined by the median percent survival in days of the test group compared with the untreated controls multiplied by 100 (T/C ratio) [4].

Statistical analysis. Each drug study was performed in duplicate and repeated five times. The ID_{50} (dose which inhibited 50% of colony formation compared with control) was extrapolated by plotting mean values for each dose–response experiment as a linear regression. In the cation blockage and rescue experiments, mean values for each dose–response experiment were compared for significance by Student's *t*-test at the OP concentrations tested.

Results

Three human cell lines (K-B2, K-T1, SUP-T1) and the mouse lymphocytic leukemia cell line (P388) were used as the source of malignant lymphoid cells [4, 16, 18]. The growth and cell surface characteristics of the human cell lines are illustrated in Table 1. Each cell line was established from a child with late stage disease who was clinically resistant to multiagent chemotherapy [16]. The sensitivity of each cell line to conventional antineoplastic drugs (vincristine, doxorubicin, prednisone, methotrexate, cytosine arabinoside) has been determined, and the drug resistance of these cell lines to the vinca alkaloids has been reported previously [17]. The malignant lymphoid cells were resistant to chemotherapy; the ID_{50} was high

Table 1. Cell surface and growth characteristics of human cell lines

Cell line	Pathologic findings	Cell surface markers[a]	Doubling time	Cloning effeciency[b]
K-B2	Burkitt's lymphoma	SIg + SRBC − Leu 1,9 −	29 h	20%
K-T1	Acute lymphoblastic leukemia[c]	SIg − SRBC + Leu 1,9 +	50 h	12%
SUP-T1	Convoluted cell lymphoma[c]	SIg − SRBC − Leu 1,9 +	35 h	5%

[a] +, present; −, absent; SIg, surface immunoglobulin; SRBC, sheep red blood cell receptor; Leu 1,9, pan T cell markers
[b] % cloning efficiency = (number of colonies grown/number of cells seeded) × 100
[c] T cell subtype [16, 18]

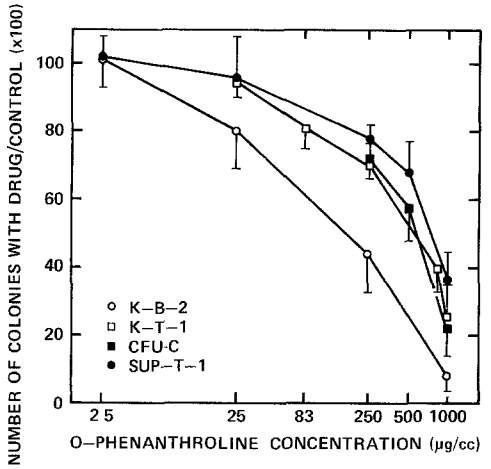

Fig. 1. Dose–response curves of cell lines and CFU-C exposed to OP for 1 h. *Points,* means of quintuplicate assays ±SEM

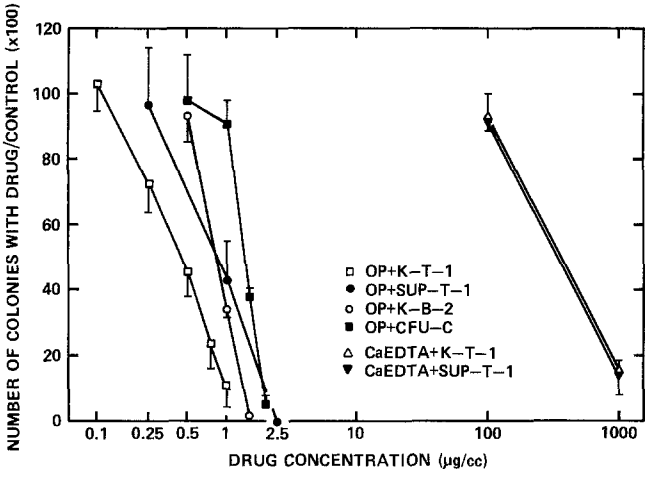

Fig. 2. Dose–response curves of cell lines and CFU-C continuously exposed to OP, or EDTA. *Points,* means of quintuplicate assay ±SEM

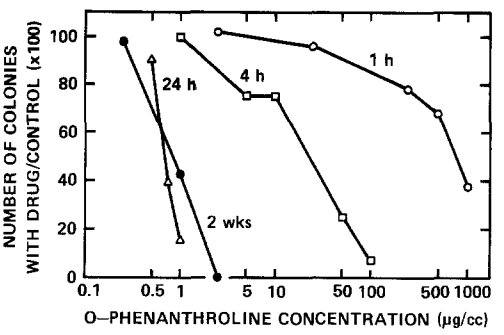

Fig. 3. Dose–response curves for SUP-T1 cells exposed to OP for 1, 4 or 24 h or continuously for 2 weeks

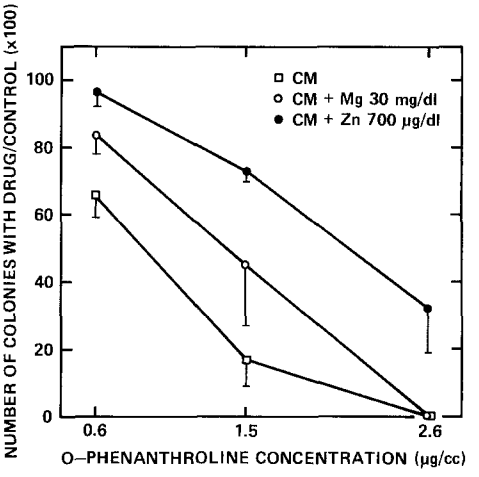

Fig. 4. Dose–response curves for SUP-T-1 cells exposed to OP continuously while incubated in complete medium *(CM)* containing Zn (35 μg/dl) and Mg (3 mg/dl) (□); CM containing Mg (30 mg/dl and Zn (35 μg/dl) (○), or CM containing Zn (700 μg/dl) and Mg (3 mg/dl) (●).

(5–50 μg/ml), and the antimyeloid effect was more severe than the antilymphoid effect of all doses tested [17]. The dose–response curve demonstrating the effect of 1 h exposure to OP on the test cells is illustrated in Fig. 1. Both the CFU-C and the cell lines showed a direct dose–response effect with escalating doses of OP. The ID_{50}s for K-B2, K-T1, SUP-T1, and CFU-C were 112, 417, 975, and 537 μg/cm^3, respectively.

The dose–response curve demonstrating the effect of continuous exposure of OP (and CaEDTA) on the test cells is shown in Fig. 2. The ID_{50}s for K-T1, SUP-T1, K-B2, and CFU-C to OP were 0.43, 0.69, 0.84, and 1.3 μg/cm^3, respectively. The relative sensitivity index (SI = x ID_{50} CFU-C ÷ x ID_{50} cell lines) for the cell lines ranged from 1.5–3.0. At 1 μg/cm^3, the three cell lines demonstrated significantly higher sensitivity to OP than did CFU-C ($P<0.05$); however, once a threshold value was reached the slope of each dose–response curve was quite steep (Fig. 2). The ID_{50}s for K-T1, SUP-T1, and CFU-C to CaEDTA were 355, 398, and 34 μg/cm^3, respectively. Thus, the two T cell lines tested were over 500 times more sensitive to a continuous exposure of OP than to CaEDTA.

The effect of various durations of OP exposure on the SUP-T1 cell line is illustrated in Fig. 3. When the OP exposure time was increased from 1 h to 24 h the maximum effect of OP was observed and a 3-log shift to the left in the dose–response curve was obtained.

Figure 4 shows the effect of supplemental cations on the dose–response curve of OP (continuous exposure) tested with SUP-T1 cells. Supplemental Zn, and to a lesser extent Mg, blocked the OP effect only at relatively low concentrations of OP. In experiments evaluating the ability of cations to rescue OP's cytotoxicity, supplemental Mg (30 mg/dl) and Zn (700 μg/dl) did not change the toxic effects of OP (results not shown).

The effect of OP on mouse lymphocytic leukemia (P388) was evaluated both in vitro and in vivo. A direct dose–response curve was obtained when P388 cells were continuously exposed to OP in vitro (ID_{50} = 0.71 μg/cm^3).

Table 2. Survival of mice inoculated with P388 and treated with OP or 5-FU

Drug	Route of drug administration	Treatment schedule	Dose (mg/kg/inj)	T/C[a]
5-FU	IP	Daily, days 1 – 5	20	167%
OP	IP	Daily, days 1 – 5	20	100%
			10	100%
			2	100%
OP	IP	Every 3 h, days 1, 5, 9	50	85%
			10	110%
			1	120%
OP	SQ	Every 3 h, days 1, 5, 9	50	109%
			10	118%
			2	105%
OP	SQ	Every 3 h, days 1 – 5	2	117%

[a] T/C = (median survival of test group/median survial of control) × 100. T/C > 127% indicates antitumor activity of the test drug while T/C < 85% indicates toxicity [19, 20]

However, OP did not prolong the survival of mice inoculated with P388 cells (Table 2). While 5-fluorouracil demonstrated appropriate antitumor activity (positive control), OP showed no effect in the prescreen (drug given daily, days 1 – 5) and no effect in treatment regimens designed to take advantage of the time-dependent nature of OP's cytotoxicity in vitro.

Discussion

Previous reports demonstrated that 1,10-orthophenanthroline (OP) was cytotoxic to both normal and malignant human T-lymphocytes [3, 21]. To determine whether OP was preferentially toxic to hematopoietic cells of the T cell lineage, we determined the effect of increasing concentrations of OP on the growth of B- and T-lymphoblasts and on normal myeloid colony-forming cells (CFU-C). Drugs which are selectively toxic to T-lymphocytes would be clinically useful as specific chemotherapy for T cell leukemia/lymphoma. New drug therapy is needed for these aggresive malignancies, because in patients treated with conventional chemotherapy drug resistance occurs early in the clinical course.

1,10-Orthophenanthroline was found to be an active agent, and OP had a direct dose – response effect on each cell line tested in vitro. This effect was time-dependent and a 3-log-enhanced kill occurred when the drug exposure time was increased from 1 to 24 h. The malignant cell lines tested had an $ID_{50} < 1$ μg/ml and were slightly more sensitive to OP than were L1210 cells, CCRF-CEM cells, and Ehrlich ascites cells [2, 3, 6]. However, contrary to other reports, OP's toxicity to the human cell lines was not reversible, because supplemental zinc incompletely blocked and did not rescue OP's cytotoxicity [2, 3, 6]. The inability of zinc to fully block or rescue OP's cytotoxicity may be due to rapid drug incorporation, which has been reported for Ehrlich ascites cells [6].

The cell lines used in these experiments were grown form patients with late stage disease who were clinically resistant to multiagent chemotherapy [16, 18]. The cell lines possessed drug resistance; conventional chemotherapeutic agents (i.e., doxorubicin, methotrexate) were ineffective in doses < 5 μg/ml. To compare the in vitro inhibitory effects of different chemotherapeutic agents, a ratio of a drug's effect on malignant lymphoid cells was compared with its effect on bone marrow myeloid stem cells (CFU-C). For OP, this ratio (sensitivity index, SI) was > 1, indicating some selective kill of malignant cells over CFU-C. However, the SI for EDTA was 0.1, indicating that at EDTA concentrations sufficient to kill malignant cells, severe myelotoxicity occurred [13]. Although in vitro – in vivo drug correlations are complex, in studies done with patients with AML an SI > 1 was associated with a complete response, while an SI < 1 was associated with induction failure [9, 10].

The current NCI protocol for the investigation of experimental antineoplastic agents evaluates whether the test drug can prolong the survival of mice inoculated with P388 leukemic cells [4, 19, 20]. While the mouse P388 cells were sensitive to OP in vitro, no effect was seen when OP was administered in vivo, even when schedules designed to take advantage of OP's time-dependent toxicity were used. While all current clinically useful antineoplastic drugs have passed the P388 screen, no recent drugs have been developed for cells which possess multidrug resistance.

A proposed NCI protocol for new drug screening will evaluate the effect of test drugs on the growth of human tumor stem cells in agar [14, 15, 19]. The test drugs found to have an ID_{70} less than 10 μg/ml against a panel of specific cancers will undergo further evaluation. While this assay will provide disease-specific information, it will not address the problem of drug resistance. Since OP was toxic to drug-resistant cell lines ($ID_{50} < 1$ μg/ml) and some selectivity occurred (SI > 1), OP may be a useful agent in the selective treatment of drug-resistant cells. However, since OP was ineffective in the in vivo P388 screen, bioavailability studies need to be completed to allow the design of drug regimens that will enhance OP's therapeutic index in vivo.

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